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EXHIBIT 11

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Functional Limits of Conformation, Hydrophobicity, and Steric Constraints in Prokaryotic Signal Peptide Cleavage Regions

WILD TYPE TRANSPORT BY A SIMPLE POLYMERIC SIGNAL SEQUENCE*

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These experiments examine the role of conformation, hydrophobicity, and steric constraints in the function of the prokaryotic signal peptide cleavage region. The experimental strategy involves replacement of the wild type *Escherichia coli* alkaline phosphatase signal peptide cleavage region with a series of idealized model sequences designed to epitomize the particular structural and physical variables under study. By analyzing model sequences whose conformations have been determined by physical studies, we have demonstrated that efficient transport does not depend on the structural preference of the cleavage region. Although previous studies based on Chou-Fasman analysis have suggested that the cleavage region forms a β-turn which is required for transport, our results demonstrate that either a β-turn- or α-helix-fostering sequence in the cleavage region functions indistinguishably from wild type. Furthermore, the presence of a proline residue between the core and cleavage region, although common in natural sequences, is not essential for export. Cleavage regions of varying hydrophobicities can support translocation across the inner membrane, but the placement of bulky residues at positions −1 and −3 upstream of the cleavage site abolishes processing and transport to the periplasm. By reducing the signal peptide to simplified, idealized segments, this study has identified a largely polymeric sequence, MKQST(L_{10})-(A_6), that functions equivalently to the wild type alkaline phosphatase signal peptide. This work starts to provide a basis for the design of a universal prokaryotic signal peptide that incorporates all the critical physical and structural characteristics required for transport function.

Prokaryotic proteins destined for transport out of the cytoplasm typically contain an amino-terminal extension called the leader or signal peptide which is required for export. Despite their common purpose in mediating protein transport, signal peptides have very little amino acid sequence homology, although they do share some general features. Typically 15–30 amino acids long, signal peptides can be divided into three characteristic segments: a 5–8-residue positively amino-terminal region, an 8–12-residue central hydr core, and a more polar 5–7-residue carboxyl-terminal region which is involved in specific recognition by peptidase (von Heijne, 1985a). Because of their lack mon sequence information, signal peptides are tho mediate transport based on structural and physical pr such as charge balance (von Heijne, 1986a), hydrop (Chou and Kendall, 1990), and conformation (Bri Gierasch, 1986) rather than strict sequence specificity

In this paper we explore the physical and conform characteristics governing the function of one key do the signal peptide, the cleavage region. This region is among signal peptide subsegments in its involveme specific enzyme-substrate interaction with signal pep the inner membrane protease responsible for removal lipoprotein signals during transport (Zwizinski and W 1980). Such a defined interaction may impose speci on cleavage region sequence characteristics, confor and polarity. Examination of natural presequences strates that the cleavage region is the portion of th peptide that varies least in amino acid composition an from protein to protein (von Heijne, 1985a). In pa statistical analysis of wild type sequences has sho residues with small neutral side chains such as Ala, and Thr at positions −1 and −3 preceding the clea with Ala being by far the most favored (von Heijne This sequence pattern has been suggested to form for substrate recognition by signal peptidase and has be known as the "−3, −1 rule" (von Heijne, 1984).

In addition to amino acid composition, the conform the cleavage region has also been implicated in its Several models of prokaryotic protein transport i least the transient formation of a turn or loop structu signal peptide insertion and/or cleavage (von H Blomberg, 1979; Wickner, 1980; Inouye and Halegc Engelman and Steitz, 1981; Briggs and Gierasch, 19 been proposed that the cleavage region may form which facilitates insertion and may be required for re and cleavage by signal peptidase (Rosenblatt *et* Perlman and Halvorson, 1983). A number of lines o lend circumstantial support to this notion. Sequenc of prokaryotic signal peptides reveals that they ofte a proline or another turn-promoting residue (*e.g.* G at the core-cleavage region boundary (von Heijne, 1 residue is frequently found in β-turns in globula (Chou and Fasman, 1978). Application of secondary predictive schemes to known signal sequences ofte always, predicts a β-turn in the cleavage region (Pe Halvorson, 1983). In addition, mutational studies

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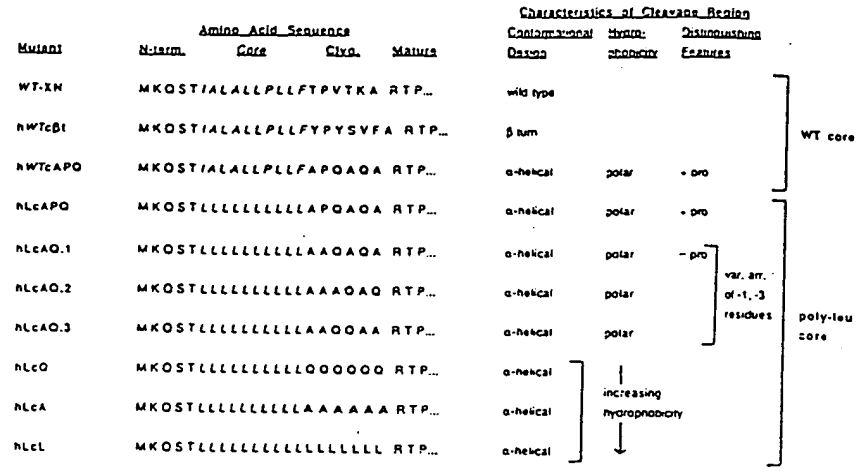

FIG. 1. Amino acid sequences and design features of cleavage region models. The sequence of the amino-terminal region is shown in *plain type*, the core region in *italics*, the cleavage region in *boldface*, and the mature protein in *plain type*. Mutants are named systematically, with the letters following the *h* designating the derivation of the core region, and those following the *c* indicating the salient features of the model cleavage region. The conformational design, hydrophobicity, and other distinguishing characteristics of each mutant are tabulated to the *right* of its sequence.

gested that the efficiency of precursor processing declines as the cleavage region decreases in β-turn probability by Chou-Fasman parameters (Vlasuk *et al.*, 1984).

In addition to these conformational considerations, the hydropathy of the cleavage region may be functionally important as well. Examination of natural sequences reveals that this region is more hydrophilic than the apolar core that precedes it, but only rarely contains charged residues (von Heijne, 1986b). During translocation, the cleavage region must traverse the interior of the membrane in contact either with the lipid bilayer or with protein(s) residing in it. However, it also has to associate with the signal peptidase active site which faces the aqueous periplasmic compartment (Zimmermann *et al.*, 1982). These different environments may each impose their own restrictions on cleavage region polarity.

The purpose of this paper is to define the critical physical and structural characteristics of a functional prokaryotic signal peptide cleavage region. To do this, a series of mutants was constructed using cassette mutagenesis to replace the native *Escherichia coli* alkaline phosphatase signal peptide cleavage region with a series of idealized nonnatural model sequences meant to epitomize the particular variables under study. These mutants were designed to evaluate in a systematic way the functional limits of conformation and hydrophobicity in the cleavage region, as well as to test the validity of the −3, −1 rule and the importance of proline. Fig. 1 shows the amino acid sequences and design features of all the cleavage region mutants. The conformational models incorporate sequences whose structures have already been determined by physical studies. The β-turn model derives from an NMR study of turn-forming peptides from Wright and colleagues (Dyson *et al.*, 1988), and the model helical sequences were obtained from circular dichroism studies of amino acid homo- and copolymers (Krull *et al.*, 1965; Auer and Doty, 1966a, 1966b; Ferretti and Paolillo, 1969).

Based on this approach, we have found that a potential β-turn structure in the cleavage region is not required for translocation, processing, and export. Either an α-helix- or β-turn-fostering sequence in the cleavage region can function with an efficiency indistinguishable from wild type, irrespective of the presence of a proline residue. A relatively broad range of hydrophobicities can support the membrane translocation step of export. However, subsequent processing and release into the periplasmic space requires compliance with the −3, −1 rule. By reducing the signal peptide to simplified, idealized segments that still retain virtual wild type transport effectiveness, these studies start to provide a basis for the design of a universal idealized signal peptide that incorporates all the critical physical and structural characteristics required for transport function.

MATERIALS AND METHODS

Bacterial Strains and Media

The strain used in these studies is *E. coli* AW1043 (Δ*lac galU galK* Δ(leu-ara) phoA-E15 proC::Tn5). This strain contains a partially deleted alkaline phosphatase gene and a wild type *phoR* regulatory gene (Inouye *et al.*, 1981; Ghosh *et al.*, 1986). For site-directed mutagenesis, the male derivative AW1043F'Tet (Δ*lac galU galK* Δ(leu-ara) phoA-E15 proC::Tn5 F'Tet) was used.

Bacteria were propagated in LB medium or plates containing 50 μg/ml kanamycin and 250 μg/ml ampicillin (Maniatis *et al.*, 1982). For transport studies, cells were cultured in MOPS[1] medium (Neidhardt and Bloch, 1974) with antibiotics as above, under low phosphate conditions (100 μM) or zero phosphate to induce alkaline phosphatase expression.

Construction of Cassette Mutagenesis Vectors

WT-XN—*WT*-XN is a modified form of WT CASS3. WT CASS3 contains the alkaline phosphatase structural gene *phoA* in which unique *Sal*I and *Bss*HII sites bracket the core coding region (Kendall and Kaiser, 1988). *WT*-XN contains two additional novel and unique restriction sites, *Xho*I and *Nhe*I, at codons 8–9 and 23–24 of mature alkaline phosphatase, respectively (Fig. 2). The *Xho*I site maintains the wild type codons for leucine and glutamic acid at this position; the *Nhe*I site changes the wild type arginine codon at position 24 to a serine. The *WT*-XN cassette vector was constructed by site-directed mutagenesis essentially as described (Kendall and Kaiser, 1988; Butler-Ransohoff *et al.*, 1988). Creation of the new *Nhe*I and *Xho*I sites was verified by restriction analysis using these enzymes (Maniatis *et al.*, 1982) as well as by direct DNA sequencing (Sanger *et al.*, 1977). Expression levels and transport properties of *WT*-XN were indistinguishable from WT CASS3.

10L-XN—The 10L-XN cassette vector was created by removal of the wild type *Sal*I/*Bss*HII 52-mer from *WT*-XN followed by LGT isolation of the remaining large vector. A new 52-mer was obtained by *Sal*I/*Bss*HII digestion of a WT CASS3 derivative containing a core region composed of 10 leucines including a *Hpa*I site in the final leucine codon (Chou and Kendall, 1990). After separation by 8% PAGE and staining with ethidium bromide, the polyleucine-encoding 52-mer was purified and ligated to the host *WT*-XN cassette vector essentially as described in Kendall and Kaiser (1988).

[1] The abbreviations used are: MOPS, 4-morpholinopropanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

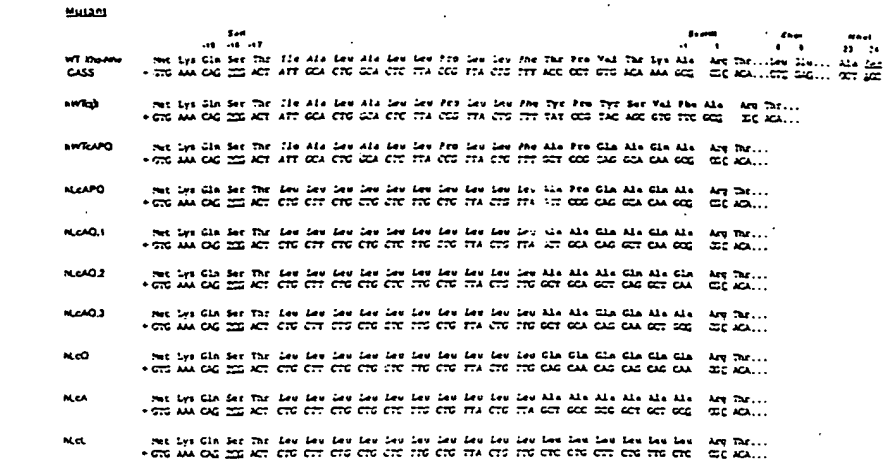

FIG. 2. Amino acid and DNA sequences of cleavage region mutants along with WT *Xho-Nhe* CASS. WT *Xho-Nhe* CASS contains unique *Sal*I, *Bss*HII, *Xho*I, and *Nhe*I sites which were introduced into the wild type alkaline phosphatase structural gene as described under "Materials and Methods." Residues at these restriction sites that differ from wild type are *underlined*. All results pertaining to "wild type" in this paper refer to WT *Xho-Nhe* CASS. The amino acid and nucleotide sequences of the amino-terminal region are shown in *plain type*, the core region in *italics*, the cleavage region in *boldface*, and the mature protein in *plain type*. For clarity, only the + strand of the DNA sequence has been shown. Amino acids are numbered by counting forward from the first residue of the mature protein (+1, +2, . . .) and back into the signal peptide from the first residue upstream of the putative cleavage site (−1, −2, . . .).

Construction of Cleavage Region Mutants by Cassette Mutagenesis

Mutants h*WT*cdt and h*WT*cAPQ were constructed by cassette mutagenesis using the *Sal*I and *Bss*HII sites of the *WT*-XN vector as described (Kendall and Kaiser, 1988). All other cleavage region models were constructed using the 10L-XN cassette vector following the strategy of Chou and Kendall (1990). Each mutant construct was used to transform *E. coli* strain AW1043. The DNA and amino acid sequences of the resultant constructs are shown in Fig. 2. Plasmid DNA from the ampicillin-resistant transformants was prepared, and the mutant sequences were verified by restriction analysis and direct DNA sequencing (Sanger *et al.*, 1977).

Transport Studies

Periplasmic Lysozyme-EDTA Cell Fractionation—*E. coli* cultures harboring mutant plasmids were grown at 37 °C in MOPS containing 100 μM phosphate, 50 μg/ml kanamycin, and 250 μg/ml ampicillin. Cells were harvested in the logarithmic phase of growth, washed, and resuspended in MOPS with zero phosphate supplemented with amino acids at 2 μg/ml minus methionine. After resuspension, cells were further diluted 1:10 in this same medium prior to labeling with 42 μCi of [^{35}S]methionine for 90 s. For whole-cell samples, labeling was stopped by addition to an equal volume of ice-cold 10% trichloroacetic acid. Precipitates were washed twice in cold acetone and air-dried. Protein was resolubilized by boiling for 3 min in 10 mM Tris, pH 8, 1% SDS, 1 mM EDTA and then diluted by addition of 50 mM Tris, pH 8, 150 mM NaCl, 0.1 mM EDTA, 2% Triton X-100. For periplasmic samples, incorporation of label was stopped by rapid cooling on ice. Aliquots of labeled cultures were then washed in 30 mM Tris, pH 8, and resuspended in 0.5 M sucrose, 30 mM Tris, pH 8, containing 1 mM EDTA and 20 μg/ml freshly dissolved lysozyme. After incubation at room temperature for 20 min, samples were centrifuged, and the supernatants containing the periplasmic fraction were passed through a 0.22-μm filter prior to dilution with 50 mM Tris, pH 8, 150 mM NaCl, 2% Triton X-100, 0.1 mM EDTA. Whole cell and periplasmic samples were then subjected to immunoprecipitation using 2.5–5 μl of rabbit anti-alkaline phosphatase antiserum (Kendall and Kaiser, 1988) essentially according to the method of Ito *et al.* (1981).

Pulse-Chase Analysis—Bacteria were subcultured, washed, and resuspended as described above. In certain cases cells were further diluted 1:10 prior to labeling as outlined in the periplasmic cell fractionation protocol. Cells were labeled with 42 μCi of [^{35}S]methionine for 40 s at 37 °C and then chased with 4 mg/ml nonradioactive methionine for 30 s, 1, 5, and 15 min. The chase was terminated by addition of an equal volume of ice-cold 10% trichloroacetic acid. The resultant precipitates were washed with cold acetone, dried, and resolubilized in 10 mM Tris, pH 8, 1% SDS, 1 mM EDTA. Resolubilized proteins were then diluted in 50 mM Tris, pH 8, 150 mM NaCl, 2% Triton X-100, 0.1 mM EDTA prior to immunoprecipitation using a rabbit anti-alkaline phosphatase antiserum as described above.

Protease Protection Experiments—This protocol followed the method of Li *et al.* (1988) as modified by Chou and Kendall (1990). Cells were prepared and diluted 1:10 as described in the periplasmic EDTA cell fractionation protocol above. In addition to the periplasmic and periplasmic samples, 2 additional aliquots of cells were prepared and treated with lysozyme and EDTA. After 20 min, proteinase K was then added to a concentration of 25 μg/ml in the presence or absence of 0.2% CHAPS (Boehringer Mannheim) or an equal volume of water. After incubation for 20 min on ice and then 15 min at room temperature, ice-cold trichloroacetic acid was added to a final concentration of 10%. Trichloroacetic acid precipitates were then processed as in the pulse-chase protocol above.

SDS-PAGE and Autoradiography—Immunoprecipitated alkaline phosphatase was run on Laemmli SDS-PAGE (Laemmli, 1970), subjected to autoradiography as described (Kendall and Kaiser, 1988).

BCIP Plate Assays—Mutants were plated on MOPS agar containing 40 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) under low phosphate conditions (100 μM KH_2PO_4) to induce phosphatase expression. The colored reaction product from hydrolysis of BCIP confers a blue color to colonies containing alkaline phosphatase activity. As a negative control, cells are also plated on media containing high phosphate levels (10 mM KH_2PO_4) to repress alkaline phosphatase activity. All colonies are pale under these conditions, demonstrating that the blue color observed in these assays arises essentially from the action of alkaline phosphatase.

Amino-terminal Radiosequencing by Automated Edman Degradation—Alkaline phosphatase from the signal peptide mutants was radiolabeled and isolated for Edman degradation (Edman and Begg, 1967) essentially as in Kendall and Kaiser (1988) except for the following variations. In hLcL, hLcQ, and hLcAQ.3 incorporation of label was quenched by addition of an equal volume of 100% trichloroacetic acid to ensure that the amino-terminal methionine of the alkaline phosphatase precursor was deformylated. After 15 and 30 min at room temperature, protein was precipitated with 10% trichloroacetic acid. Precipitates were acetone-washed and resolubilized as in the pulse-chase protocol above prior to PAGE and transfer to Immobilon (Millipore) (Matsudaira, 1987) as described in Kendall and Kaiser (1988). In the case of mutants the amino-terminal sequence was determined by high pressure liquid chromatography identification of phenylthiohydantoin

tives obtained by automated Edman degradation. All sequencing was performed on an Applied Biosystems gas phase sequencer (Model 470) or pulsed-liquid sequencer (Model 477) at The Rockefeller University Protein Sequencing Facility.

RESULTS

Design of Cleavage Region Models—Fig. 1 shows the amino acid sequences and design characteristics of all the model cleavage regions tested in this study. Several general principles were used in the design of these mutants. Unless specifically intended otherwise, residues chosen at each position conformed to "permitted" residues as judged by the statistical analysis of von Heijne for prokaryotic signal peptides (von Heijne, 1986b). The positioning of proline when present, as well as the length of the model cleavage regions, also conformed to the limits of variation found in natural sequences.

To generate the different conformational models in this study, complementary strategies were used. Unlike previous approaches which have inferred conformation based on Chou-Fasman analysis (Vlasuk *et al.*, 1984; Duffaud and Inouye, 1988; Nothwehr and Gordon, 1989; Yamamoto *et al.*, 1989), we have used sequences whose conformations have already been determined by direct physical studies. The β-turn model incorporates a sequence derived from an NMR study on reverse turn-forming peptides from Wright and coworkers (Dyson *et al.*, 1988). Because short peptides usually do not form stable, identifiable secondary structures under aqueous conditions, it is difficult to find conformationally characterized sequences for modeling β-turn structures. Of the turn-forming peptides which have been identified previously, many contain D-amino acids or covalent cross-links (Rose *et al.*, 1985). Wright's study is unusual in identifying a family of short synthetic peptides composed of natural amino acids that form significant β-turn structure in aqueous solution. The sequence chosen for our work, YPYSV, was the one candidate from this study that showed the strongest turn character while still conforming as closely as possible to "permitted" residues for positions −7 through −3 of a prokaryotic cleavage region. After the proposed turn-forming sequence, two amino acids (Phe at −2 and Ala at −1) were added to generate a cleavage site predicted to be acceptable to signal peptidase (von Heijne, 1984; Kuhn and Wickner, 1985).

The putative α-helical sequences were designed according to the principles of Kaiser and Kezdy (1983) in which simplified peptide models are constructed using a minimum complement of amino acids but maintaining the desired hydrophobicity patterns and expected conformation. In this way, idealized structures can be made that epitomize the essential physical properties of the wild type peptides yet minimize homology with natural sequences. Our models were designed to vary in hydrophobicity yet retain the same conformational preference. Only leucine, alanine, and glutamine were used to compose these sequences. These residues were chosen because they differ significantly in polarity, yet all display a preference for α-helix formation by circular dichroism measurements (Krull *et al.*, 1965; Auer and Doty, 1966a, 1966b; Ferretti and Paolillo, 1969). Leucine, alanine, and glutamine represent the widest extremes of polarity combined with the strongest helical potential among residues that are naturally found at most positions in wild type cleavage regions (von Heijne, 1986b).

Because of the possible impact of neighboring amino acids on the structure of the model cleavage regions, we varied their amino acid context by placing them next to two different hydrophobic cores: first, the wild type alkaline phosphatase core region, and second, an idealized polymeric 10-leucine core designed to optimize formation of a hydrophobic α-helix. A polyleucine core has been shown previously to perform extremely efficiently in processing and transport (Kendall *et al.*, 1986). By placing this core region upstream from model sequences with a high propensity to form regular α-helical structures, a helical conformation should be stabilized and propagated from the core region through the cleavage site.

In addition to evaluating the importance of hydrophobicity and conformation, model sequences were also designed to test the role of specific amino acids in the function of the cleavage region. In particular, a proline residue is commonly found near the beginning of prokaryotic cleavage regions (von Heijne, 1986b). The experiments presented here endeavor to test whether proline is an essential component of prokaryotic cleavage regions by evaluating its functional importance when placed within simplified, idealized model sequences. In the actual design of the proline-containing sequences, the Pro residue was placed at position −5 where it occurs most commonly in natural prokaryotic signal peptides. When an amino acid was needed to replace proline in a specific model, alanine was chosen because of its small size, neutrality, modest hydrophobicity, and common occurrence at all positions in natural cleavage regions (von Heijne, 1986b).

In addition to analyzing the importance of proline, these models also incorporate a direct test of the −3, −1 rule which states that small, neutral residues are required at these positions for recognition and cleavage by signal peptidase (von Heijne, 1984). This was done by selectively violating or maintaining the pattern of "permitted" residues at these two positions while keeping the amino acid composition of the model cleavage regions the same.

Transport Studies—All the mutants in this study were constructed by cassette mutagenesis using the *WT*-XN cassette vector as described under "Materials and Methods." The cleavage region models were first tested for overall function by evaluating their ability to promote the final stage of transport, namely delivery of mature alkaline phosphatase into the periplasmic space. This was done by analyzing the periplasmic contents of *E. coli* released by lysozyme-EDTA cell fractionation. As shown in Fig. 3, most of the cleavage region mutants were competent to export alkaline phosphatase to the periplasm. Remarkably, mutants incorporating either the model β-turn sequence (Dyson *et al.*, 1988) or the helix-fostering polyalanine sequence supported transport of

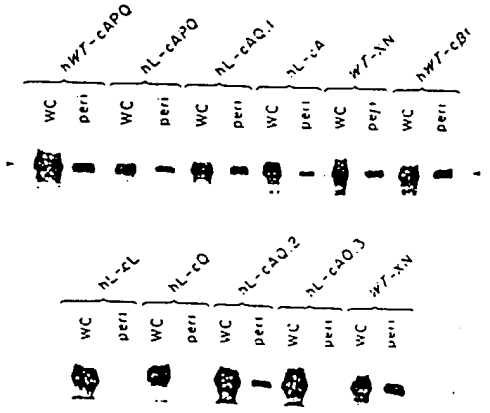

FIG. 3. Lysozyme-EDTA cell fractionation of cleavage region mutants. Cell fractionation was performed as described under "Materials and Methods." An *arrowhead* marks the position of the mature form of alkaline phosphatase in whole cell (*WC*) and periplasmic (*peri*) fractions in mutants h*WT*cAPQ, hLcAPQ, hLcAQ.1, hLcA, *WT*-XN, and h*WT*cBI. See legend to Fig. 1 for discussion of electrophoretic mobilities of mutants hLcL, hLcQ, and hLcAQ.3.

the mature enzyme. The only export-incompetent mutants were: hLcL, the polyleucine cleavage region, hLcQ, the polyglutamine cleavage region, and hLcAQ.3, the AAQQAA cleavage region. Within the limits of detection of this experiment, these three mutants generate no alkaline phosphatase in the periplasmic fraction.

Because release of exported proteins into the periplasm usually depends on cleavage of the signal peptide (Koshland *et al.*, 1982), pulse-chase analysis was used to test the efficiency of precursor processing in these mutants. As demonstrated in Fig. 4, most of the mutants were processed rapidly, with only mature alkaline phosphatase evident even at the earliest time point. This result corroborates the cell fractionation experiments (Fig. 3) which also demonstrated only mature periplasmic enzyme from most mutants after 90 s of labeling. However, pulse-chase analysis coupled with amino-terminal radiosequencing to verify identifications (see below, Fig. 5) reveals that the same mutants which were unable to be transported to the periplasm (hLcL, hLcQ, and hLcAQ.3) also failed to be processed. Since these mutants did not show both precursor and mature forms at any point during the chase, and because electrophoretic mobility changes due to certain types of mutations have been observed previously (Kendall *et al.*, 1986 and references cited within), the amino-terminal sequencing analysis was necessary to confirm that the bands observed did indeed correspond to the precursor form of the enzyme. Sequencing analysis did confirm, for example, that mutant hLcAQ.3 was present only in the precursor form although it migrated faster than the wild type. Collectively, these results demonstrate close coupling between signal peptide removal and release of the mature form in all the cleavage region mutants. Signal peptides are cleaved either rapidly or not at all, and only the former case results in periplasmic localization.

To confirm the processing behavior of these mutants and to verify the exact site of cleavage when it occurred, we performed amino-terminal radiosequencing of mutants hLcL, hLcQ, hLcAQ.2, hLcAQ.3, and hLcA. Fig. 5 shows the results obtained from scintillation counting of phenylthiohydantoin derivatives from the first four of these mutants. Radiose-

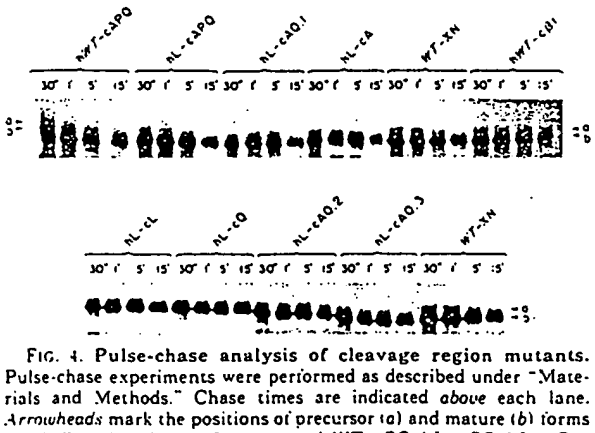

FIG. 4. Pulse-chase analysis of cleavage region mutants. Pulse-chase experiments were performed as described under "Materials and Methods." Chase times are indicated *above* each lane. *Arrowheads* mark the positions of precursor (*a*) and mature (*b*) forms of alkaline phosphatase for mutants h*WT*cAPQ, hLcAPQ, hLcAQ.1, hLcA, *WT*-XN, and hWTcdt. Amino-terminal sequencing of mutants in the *lower panel* verified that hLcL, hLcQ, and hLcAQ.3 remain uncleaved precursors, whereas hLcAQ.2 is processed (Fig. 5). The faster-than-expected migration of the three unprocessed mutants compared with the wild type alkaline phosphatase precursor (*top panel, WT*-XN (*a*)) may reflect differences in SDS binding due to their greater hydrophobicity arising from the polyleucine core. Similar changes of electrophoretic mobility in mutant precursors have been observed in other systems (Vlasuk *et al.*, 1984; Kendall *et al.*, 1986).

quencing data from mutants hLcL and hLcQ are con with their identification as uncleaved precursors (Fi and *B*). The next group of mutants, AQ.1, AQ.2, anc was designed to vary the pattern of amino acids at −3 to assess the importance of residues at these positic cleavage efficiency and specificity. Each has the same acid composition and differs only in residue order. which contains a classically permitted Ala-*X*-Ala co tion, is efficiently cleaved and transported (Figs. 3 anc contrast, AQ.2 contains forbidden glutamines at −1 a but has permitted alanine residues one position remo −2 and −4. This mutant is also processed efficientl NH_2-terminal sequencing demonstrates cleavage a shifted site: processing now occurs between the last and glutamine instead of at the usual wild type cleavag just before the arginine (Fig. 5*C*). Finally, mutant AQ.3 differs only by the position of one glutamine) was desi offer no combination of permitted residues at any l within the cleavage region. As expected, amino-termi quencing shows that this mutant indeed fails to be c (Fig. 5*D*). By testing sequences of identical amino aci position, hydrophobicity, and conformational preferen experiments confirm that it is the steric characteris potential −3 and −1 residues that constrain the ch cleavage site. These findings are in good agreement recently published study (Fikes *et al.*, 1990) analyz effects of multiple amino acid substitutions at −3 and processing of the signal peptide of maltose-binding pr

Our results demonstrate that signal peptidase has flexibility in its choice of cleavage point, and that w certain window, it can make use of alternate sites con acceptable −3, −1 residues if the usual site has been re unsuitable. However, there is a limit to this flexibi demonstrated by the processing behavior of the poly cleavage region mutant. Given that alanine is the most i residue at −3 and −1, a six-alanine cleavage region several potential targets for signal peptidase. However, terminal sequencing of this mutant demonstrates th material which is successfully processed and transpo the periplasm is all cleaved uniquely at the canonic (Table I). Although von Heijne (1984) has suggested t sequence of the cleavage region and early mature por transported proteins has evolved to avoid the poten ambiguous processing, our results experimentally ver other factors must also contribute to the mainten cleavage specificity. On a statistical basis, distance fr end of the core region has been proposed as another p determinant of cleavage site (von Heijne, 1984), and th account for the surprising fidelity of the cleavage even polyalanine mutant.

The data presented above demonstrated that n hLcL, hLcQ, and hLcAQ.3 were incompetent to proc export alkaline phosphatase. Therefore, we wished to mine more precisely at what step in the transport p they were impaired. Sodium hydroxide cell fractic (Russel and Model, 1982) indicated that these mutan capable of association with the membrane (data not suggesting that they might at least be able to partic the early stages of transport. Therefore, protease pr experiments were used to assess the ability of these to translocate alkaline phosphatase across the inne brane following membrane insertion. This approac advantage of the fact that alkaline phosphatase, upo location to the periplasmic face of the inner membra extremely tightly and is strongly resistant to proteo. *et al.*, 1988). However, untranslocated alkaline phos

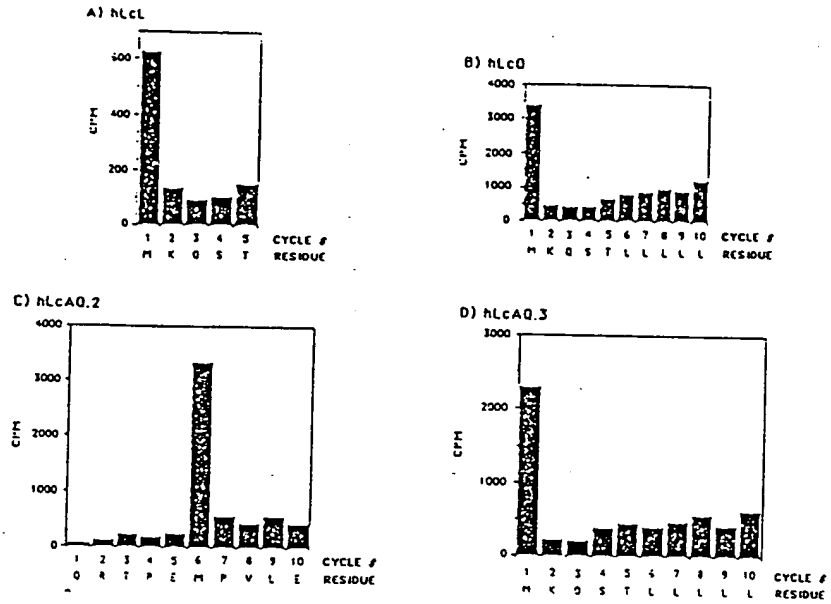

FIG. 5. Radiosequencing of cleavage region mutants hLcL, hLcQ, hLcAQ.2, and hLcAQ.3. Cells bearing mutant plasmids were labeled with [^{35}S]methionine, and their alkaline phosphatase was isolated and sequenced as described under "Materials and Methods." The *bar graph* represents the radioactivity determined for each cycle of Edman degradation. The natural amino acid sequence correlating with the experimental results is shown *below* the plot. *A, hLcL*: the peak of radioactivity at cycle 1 corresponds to the sequence of unprocessed alkaline phosphatase precursor. *B, hLcQ*: the peak of radioactivity at cycle 1 corresponds to the sequence of unprocessed alkaline phosphatase precursor. *C, hLcAQ.2*: the peak of radioactivity at cycle 6 corresponds to the sequence of alkaline phosphatase cleaved just after the "permitted" combination of alanines at −4 and −2, resulting in an extra glutamine residue at the mature amino terminus. A *caret* marks the point of cleavage by signal peptidase. *D, hLcAQ.3*: the peak of radioactivity at cycle 1 corresponds to the sequence of unprocessed alkaline phosphatase precursor.

TABLE I

Amino-terminal sequencing of mutant hLcA

The sequence shown derives from high pressure liquid chromatography identification of phenylthiohydantoin derivatives obtained by automated Edman degradation of periplasmic alkaline phosphatase. This material was isolated from radiolabeled cells by lysozyme-EDTA cell fractionation, immunoprecipitation, SDS-PAGE, and transfer to Immobilon as described under "Materials and Methods." An additional minor component was also identified containing the identical sequence minus the amino-terminal arginine. This species constituted less than 20% of the material analyzed per cycle and corresponds to an isozymic variant of alkaline phosphatase. The prevalence of this isozyme varies according to growth conditions and is known to be generated by post-processing removal of the first arginine of the mature sequence by the arginine-specific protease iap (Laforet *et al.*, 1989). These amino-terminal sequencing results indicate the signal peptide cleavage event in mutant hLcA occurred exclusively at the canonical wild type site; no processing occurred upstream at other sites within the polyalanine cleavage region itself.

Cycle	Major residue
1	Arg
2	Thr
3	Pro
4	Glu
5	Met
6	Pro
7	Val
8	Leu
9	Glu
10	Asn

that is still in the cytoplasm cannot fold properly (Boyd *et al.*, 1987) presumably because of the reducing environment there (Summers and Knowles, 1989) and consequently is

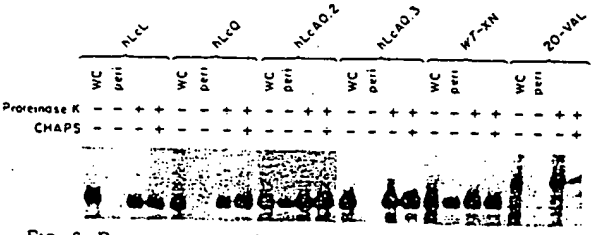

FIG. 6. Protease protection experiment. Mutants hLcL, hLcQ, hLcAQ.2, hLcAQ.3, *WT*-XN, and 20-VAL were analyzed for translocation of alkaline phosphatase to the periplasmic space. Cells were fractionated with lysozyme and EDTA then subjected to treatment with proteinase K with or without the detergent CHAPS as described under "Materials and Methods." Periplasmically oriented alkaline phosphatase is resistant to protease in the presence or absence of CHAPS, whereas cytoplasmic alkaline phosphatase is resistant in the absence of CHAPS but susceptible when detergent is added.

vulnerable to proteolytic attack. To distinguish between these two different orientations experimentally, cells are treated with lysozyme and EDTA to disrupt the cell wall and outer membrane. Then proteinase K is added with or without the detergent CHAPS which permeabilizes the inner membrane, allowing the protease access to the cytoplasm. Cytoplasmically oriented alkaline phosphatase should be protected from proteinase K by the inner membrane but susceptible when detergent is added. Conversely, periplasmically oriented alkaline phosphatase should be resistant to protease in both the presence and absence of detergent.

Fig. 6 shows the results from this experiment. All the cleavage region mutants fail to be degraded by proteinase K whether or not CHAPS is present, indicating that they have

been successfully translocated to the periplasmic side of the membrane. As a control, we confirmed that an alkaline phosphatase signal peptide mutant (20-VAL), which is known to remain in the cytoplasm as an uncleaved precursor (Chou and Kendall, 1990), is susceptible to proteolysis when detergent is present (Fig. 6, *20-VAL*).

These results demonstrate that although mutants hLcL, hLcQ, and hLcAQ.3 are defective in processing and release into the periplasm, they are competent to translocate alkaline phosphatase across the inner membrane. This conclusion was further corroborated by plating these mutants on agar containing the chromogenic alkaline phosphatase substrate BCIP. Under conditions inducing alkaline phosphatase expression, all the cleavage mutants generated blue colonies indistinguishable from wild type. Because only mutants that have been successfully translocated toward the periplasmic space are enzymatically active (Boyd *et al.*, 1987), this finding provides additional confirmation that these mutants can support transfer across the bilayer.

DISCUSSION

In this paper we have tested the limits of hydrophobicity, conformation, and steric constraints in the prokaryotic cleavage region that can support protein translocation, processing, and export. This was done by designing model cleavage regions that meet the important criteria for formation of particular secondary structures and epitomize other desired physical properties. By analyzing the transport competence of these simplified, idealized sequences, we have demonstrated that efficient export does not depend on the conformational preference of the cleavage region. Model sequences designed to optimize either a β-turn or an α-helical conformation in the cleavage region functioned as efficiently as wild type. Furthermore, the presence of a proline residue between the core and cleavage region, although common in natural sequences, is not essential for transport. Cleavage regions of varying hydrophobicities can mediate translocation across the inner membrane but do not permit processing and transport into the periplasmic space if they violate the −3, −1 rule.

It has previously been suggested on the basis of Chou-Fasman predictive analysis that the cleavage region forms a β-turn which is required for export (Perlman and Halvorson, 1983; Vlasuk *et al.*, 1984; Duffaud and Inouye, 1988). Our work has tested this hypothesis by incorporating conformationally characterized model sequences into the cleavage region of *E. coli* alkaline phosphatase. The equivalent export efficiency of a sequence known to have strong β-turn character and a series of α-helical sequences demonstrates that the structural potential of the cleavage region is not a critical determinant of transport function. To be sure, there is no way to state unequivocally that the sequences examined in this study in fact form the predicted structures *in vivo*. Secondary structure is highly dependent on the surroundings; a particular conformation which has been characterized in isolation may not adopt the same structure when inserted into a new context. Indeed, identical pentapeptides can assume totally different conformations depending on their amino acid environment (Kabsch and Sander, 1984). In addition, protein transport is a dynamic process almost certainly involving the signal in multiple conformational changes as well as interactions with different environments (Briggs and Gierasch, 1986). These factors in themselves may profoundly influence the signal peptide's repetoire of functionally relevant secondary structures. Nevertheless, it is notable that the β-turn peptide and some of the helical sequences used here display remarkable conformational stability under a variety of conditions as determined by physical studies (Auer a 1966a, 1966b; Ferretti and Paolillo, 1969; Dyson *et* In fact, the intrinsic tendency to adopt the observe mations is so high for some of these sequences, it suggested that they may form them preferentially different flanking amino acid environments (Dys 1988; Marqusee *et al.*, 1989).

These studies have also demonstrated no funct quirement for a proline residue in the cleavage region. is commonly found at the boundary between the cleavage region in natural prokaryotic signal sequen Heijne, 1986b), and previous studies in prokaryotic including alkaline phosphatase, have shown that an proline substitution in wild type cleavage regions precursor processing (Koshland *et al.*, 1982; Kuhn a ner, 1985; Kadonaga *et al.*, 1985; Kendall *et al.*, 1 translocation (Kendall *et al.*, 1990). Rather than changes in conformational preference, this effect m simply from changes in hydrophobicity resulting from ment of proline by different residues. In our present the replacement of proline with alanine, a residue com in hydrophobicity (reviewed in von Heijne, 1985b), p processing and transport efficiency. Thus proline, wh mon in naturally occurring cleavage regions, does not be a required component of an idealized prokaryot peptide sequence.

Proline is also found frequently in eukaryotic regions (von Heijne, 1986b). Two recent studies hav that the presence and position of proline in the regi.n can affect the processing and transport of pre(Δpro)apolipoprotein A-II *in vitro* (Nothwehr and 1989) and the secretion of human lysozyme in yeas chicken-derived signal sequence (Yamamoto *et al.*, 19 greater apparent importance of proline in these stu reflect the vastly different systems employed. There nificant distinctions between the characteristics of eu *versus* prokaryotic signal peptides (Watson, 1984; vo and Abrahmsen, 1989) as well as growing evidence initial events in the export process may differ in e (Summers *et al.*, 1989). In addition, these results n reflect the types of mutations examined. Individual substitutions influence a number of physical variat their effects may derive from complicating steric ar tural influences from different neighboring residues *et al.*, 1990).

Cleavage and release does demand the presence side chains at positions −3 and −1 as predicted by vo (1984). Comparison of mutants hLcAQ.1, AQ.2, ar which have the same amino acid composition, hydrop and expected conformational preference, shows that cleavage and export depends on the availability of small amino acids spaced one residue apart. How addition to this configuration, other parameters are to define the preferred cleavage site, as demonstrate polyalanine mutant hLcA. Although presenting sign dase with multiple pairs of alanines, this mutant was only at the wild type site. This result indicates that ac factors such as distance from the end of the core r well as characteristics of the mature amino termin also participate in determining the exact choice of site (von Heijne, 1984). The physical dimensions and ity of the bilayer and the disposition of signal pept the membrane are also likely to constrain which b available for cleavage *in vivo*.

While the results presented above demonstrate amino acids at positions −3 and −1 are strongly cons

they also show that the residues at other positions may be less restricted than was previously thought. For example, our β-turn model incorporates two tyrosines which almost never occur in natural cleavage region sequences (von Heijne, 1986b). However, this tyrosine-containing mutant was transported as effectively as the native wild type sequence. It has been proposed that the specific sequence characteristics of natural signal peptides may have evolved to satisfy functional requirements other than transport efficiency, such as control of expression levels (Kaiser *et al.*, 1987). Thus, the avoidance of aromatic residues in natural cleavage regions may have more to do with other factors such as codon usage or regulation of transcription and/or translation, as opposed to transport *per se*.

To evaluate the role of hydrophobicity in cleavage region function, mutants hLcL, hLcA, and hLcQ were designed. By incorporating homopolymeric stretches of glutamine, alanine, or leucine downstream from a polyleucine core region, these models were intended to have the same high helical tendency but to differ widely in polarity. While the moderately hydrophobic polyalanine model was processed and exported efficiently, the hydrophilic polyglutamine and strongly apolar polyleucine models failed to be cleaved and transported to the periplasm. Interestingly, these mutants were capable of translocation across the inner membrane, indicating that the polarity of this subsegment did not influence passage of the mature protein across the inner membrane.

These hydrophobicity models all contain a 10-leucine core region. Previous studies from our laboratory have demonstrated that a signal peptide containing a polyleucine core functions extremely efficiently and generates less precursor at early time points in pulse-chase experiments than the wild type alkaline phosphatase signal (Kendall *et al.*, 1986). The polyleucine core is highly hydrophobic, and this characteristic is thought to be a major determinant of protein translocation across the membrane (Thom and Randall, 1988; Chou and Kendall, 1990). Thus, the processing efficiency of mutants incorporating the polyleucine core may reflect rapid delivery to, as well as recognition by, signal peptidase. Likewise, the failure of polyleucine-containing mutants hLcAQ.3, hLcQ, and hLcL to be cleaved probably arises from their violation of the −3, −1 rule rather than impaired access to the enzyme.

One of the hydrophobicity models, hLcL, with its positively charged amino terminus, apolar stretch of 16 leucines, and lack of classical −3, −1 residues, has many features reminiscent of Class II transmembrane proteins (reviewed in von Heijne, 1988). Previous work by Davis and Model (1985) has shown that 16 residues is the minimum length required for formation of a hydrophobic membrane anchor. In fact, sodium hydroxide cell fractionation and protease protection analysis of hLcL demonstrated that this uncleaved polyleucine-containing precursor is translocated but remains associated with the membrane. This result is consistent with previous work from our laboratory showing that an uncleaved alkaline phosphatase signal peptide containing 20 leucines results in a translocated but membrane-anchored precursor (Chou and Kendall, 1990).

By designing simplified, idealized model sequences, this study has identified a largely polymeric sequence, MKQST $(L_{10})(A_6)$, that functions comparably to the wild type signal peptide. A model cleavage region composed of six alanines may be uniquely suited to satisfy the various functional requirements imposed on natural cleavage regions *in vivo*. Alanine is already the most common amino acid found at virtually all positions in wild type cleavage regions (von Heijne, 1986b). Its small size is favored for enzyme-substrate recognition by signal peptidase according to the −3, −1 rule. In addition, despite the strong helix-fostering properties of alanine-based peptides *in vitro* (Marqusee *et al.*, 1989), alanine's small side chain also affords conformational flexibility which may be demanded for "induced fit" type interactions with components of the transport machinery or with signal peptidase.

Given the efficiency of this minimal "optimized" signal peptide, the question remains why natural signals have not converged toward such a simple sequence. There may be other constraints such as codon usage, regulation, or tailoring of sequences to the transport requirements of particular proteins (Laforet *et al.*, 1989) that contribute to the maintenance of signal sequence heterogeneity *in vivo*. Although natural signal peptides may require this diversity, this study has shown that there are also universal physical and structural properties shared by all signal peptides that govern their essential function.

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